

Aromatase Activity in the Ovary and Brain of the Eastern Mosquitofish (*Gambusia holbrooki*) Exposed to Paper Mill Effluent

Edward F. Orlando,^{1,2} William P. Davis,³ and Louis J. Guillette Jr.¹

¹Department of Zoology, University of Florida, Gainesville, Florida, USA; ²Biology Department, St. Mary's College of Maryland, St. Mary's City, Maryland, USA; ³National Health and Environmental Effects Research Laboratory, Gulf Ecology Division, U.S. Environmental Protection Agency, Gulf Breeze, Florida, USA

Studies have shown that female mosquitofish living downstream of a paper mill located on the Fenholloway River, Florida, have masculinized secondary sex characteristics, including altered anal fin development and reproductive behavior. Masculinization can be caused by exposure to androgens in the water or from an alteration in aromatase activity in the fish. We hypothesized that aromatase activity would be inhibited by a component(s) of the paper mill effluent. Aromatase inhibition could masculinize the hormonal profile and, subsequently, secondary sex characteristics of the exposed females. Therefore, we predicted that ovarian and brain aromatase activity would be lower in the female mosquitofish from the Fenholloway River compared with the reference site, the Econfinia River. Adult females were collected and standard length, body mass, anal fin length, and segment number were measured. Ovarian and brain aromatase activity were determined using a tritiated water assay. Fenholloway females had masculinized anal fin development as indicated by an increase in the number of segments in the longest anal fin ray ($p < 0.0001$), yet the length of the ray did not differ between sites ($p = 0.95$). Fenholloway females exhibited higher ovarian ($p = 0.0039$) and brain ($p = 0.0003$) aromatase activity compared with reference site fish. These data do not support aromatase inhibition as the mechanism for masculinization, suggesting that the masculinization of the Fenholloway female mosquitofish is due to androgenic contaminants. Future studies should examine the relationship between aromatase enzyme activity and exposure to environmental androgens. **Key words:** altered development, aromatase, brain, endocrine disruption, gonad, masculinization, paper mill effluent. *Environ Health Perspect* 110(suppl 3):429–433 (2002).

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During the last decade, a growing literature has documented the endocrine-disruptive effects of various environmental contaminants [for review, see (1–3)]. The focus of many early studies was on the estrogenic and antiandrogenic actions of various agricultural chemicals such as dichlorodiphenyl-trichloroethane (DDT) and its metabolites, methoxychlor, and vinclozolin and industrial compounds such as polychlorinated biphenyls (PCBs), alkylphenol ethoxylates (APEs), and phthalates (4–9). To date, research has focused on the effects of environmental exposure to these chemicals on reproduction and development in wildlife and/or investigating the underlying mechanisms for some of the observed effects in laboratory and environmentally exposed wildlife species.

In laboratory studies, researchers have examined the affinity of environmental contaminants for the estrogen receptor or a chemical's ability to induce estrogenic responses such as cell proliferation or vitellogenin synthesis (10,11). A wide range of chemicals were shown to have an affinity for estrogen receptors from various species (12). In addition to the estrogenic or antiestrogenic nature of chemical contaminants, data are currently available documenting antiandrogenic (8,9), androgenic (13–15), antiprogesterone (16), and antithyroidal

actions (17–19). The mechanisms behind these actions appear varied, with receptor binding only one of a number of possible responses. For example, contaminants also appear to influence the endocrine system by altering enzymes that enable hormone synthesis (20–22), hormone metabolism (23,24), and hormone storage on plasma carrier proteins (25,26).

One enzyme whose activity appears to be altered by environmental contaminants is the steroidogenic enzyme P450 aromatase. Aromatase converts (the androgens) androstenedione or testosterone to the estrogens estrone or 17 β -estradiol (E_2), respectively (27). This enzyme has been identified in the gonad, liver, brain, and various peripheral tissues, such as fat cells in mammals, as well as in the gonad and brain of all other vertebrates including fishes and the cephalochordate, amphioxus (27–29). At least two forms of aromatase have been reported in mammals and fishes, with gonadal and brain forms coded for on two separate genes (30–32). Aromatase expression is constitutive in some tissues, but aromatase expression and activity can be altered by temperature, season, and various hormones, such as steroids (33–35). Exposure of embryonic alligators to the herbicide atrazine induces a significant increase in aromatase activity in the gonad–adrenal–mesonephros of neonates (21).

Similar responses have been reported after *in vitro* exposure of a human adrenal cell line to atrazine (36), and two other triazine herbicides, simazine and propazine, also induced aromatase activity in human adrenal and human placental choriocarcinoma cell lines (37). Such studies indicate that aromatase activity can also be affected by environmental contaminants.

Two well-documented examples of environmental exposure to endocrine-disrupting chemicals in fishes are from sewage treatment plant effluent and paper mill effluent. Researchers in Great Britain and the United States have demonstrated that exposure to sewage treatment plant effluent causes vitellogenesis, decreased testis size, and altered sex steroid concentrations in male fishes. These effects have been associated with naturally occurring estrogens (estrone and E_2) and synthetic estrogens from birth control pills (ethinylestradiol) found in the effluent (38–40).

Additional studies have documented a suite of alterations in the function of endocrine and reproductive systems and the morphological development of fishes exposed to paper mill effluent. White suckers, *Catostomus commersoni*, exposed to paper mill effluent in Lake Superior, Canada, are known to exhibit altered pituitary function, decreased plasma sex steroid concentrations, increased liver size, elevated hepatic mixed-function oxidase levels, decreased egg and gonadal size, and delayed age to sexual maturity (41–43).

Beginning in the 1980s, a number of researchers reported masculinized female fish in rivers below paper mill effluent outfalls (44–46). Early studies observed female mosquitofish (*Gambusia affinis* and *G. holbrooki*) with gonopodial development and altered reproductive behavioral patterns.

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Address correspondence to E.F. Orlando, St. Mary's College of Maryland, Biology Dept., 18952 E. Fisher Road, St. Mary's City, MD 20686-3001 USA. Telephone: (240) 895-4376. Fax: (240) 895-4996. E-mail: eforlando@smcm.edu

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Mosquitofish are viviparous and therefore have internal fertilization. Males transfer the spermatheca to females using a highly modified anal fin called the gonopodium. Gonopodial development is androgen dependent and can be induced in females exposed exogenously to androgens (47,48). It has been hypothesized that bacterial biotransformation of plant sterols in the effluent generates the androgens responsible for the observed masculinization of female mosquitofish exposed to paper mill effluent (49). Recently, it was reported that androstenedione was present after a chemical characterization of the effluent from a paper mill plant in Florida (50). Using a number of *in vitro* receptor binding and gene expression assays, our laboratory in collaboration with others has not found sufficient androstenedione concentration in Fenholloway River water samples to support the reported significant androgenic actions of this effluent, however (15).

The present study was designed to test a mechanism that would explain observations of masculinized female mosquitofish (*G. holbrooki*) living downstream of the Buckeye Paper Mill, in the Fenholloway River, Florida (impacted site). The Fenholloway River receives approximately 50 million gallons per day of effluent from the paper mill. The effluent can comprise up to 80% of the Fenholloway River volume during the drier months of the year (46,51). In contrast to the Fenholloway River, the Econfina River (reference site) has no known point sources of pollution. The rivers share the same headwaters and both terminate in the Gulf of Mexico (15). The Fenholloway River is greatly altered both physicochemically and biologically, as evidenced by its increased temperature and decreased dissolved oxygen, clarity, and species diversity of both plants and animals compared with the Econfina River.

As described above, mosquitofish collected below paper mills can exhibit masculinized characteristics, such as gonopodial development. However, not all females in the population exposed to androgenic paper mill effluent exhibit masculinization. Further, female mosquitofish showing dramatic gonopodial development are also pregnant, suggesting that the environmental androgens can alter morphology and behavior but need not preclude reproductive activity. One could hypothesize that the masculinized female anal fin was due to immersion in water containing androgenic substances that diffused across the epithelial layers overlying the fin, stimulating the cells locally to differentiate. Given the reports of altered behavior in masculinized female mosquitofish, a more likely hypothesis suggests

that environmental androgens are active systemically, not just peripherally. Thus, females would respond to exogenous androgens in a manner that could maintain an internal hormonal environment allowing reproductive cyclicity. One mechanism by which this could be achieved would be via alterations in aromatase activity of the gonad and possibly the brain. We hypothesized that a component of the river water was inhibiting aromatase activity in the female mosquitofish, thereby resulting in a masculinized hormonal profile. Aromatase inhibition could result in a decrease in estrogens and a concomitant increase in androgens, which could masculinize the hormonal profile of a female, thereby masculinizing its secondary sex characteristics. Therefore, we predicted that ovarian and brain aromatase activity would be lower in Fenholloway mosquitofish compared with that in the reference site (Econfina River) mosquitofish.

Methods

Fish Morphometrics

In this study, we tested this hypothesis by measuring P450-aromatase activity in the ovary and brains of adult female mosquitofish. Morphometrics were obtained from all 83 fish collected. We measured aromatase activity in a subset of those fish ($n = 66$) because 17 lacked follicles in their ovaries. All of the fish were reproductively active adults, and no site differences were observed in the reproductive stage of the ovaries. On 15 August 1999, all fish were captured from the Fenholloway and Econfina Rivers using a 1/8-inch drop net. Fish were transported in coolers partially filled with fresh water obtained from each site. At the lab, fish were housed at the same density in their respective river water and held for 24–72 hr under identical conditions of 14:10-hr light:dark photoperiod and 28°C water temperature.

All lab work was conducted in full compliance with the guidelines of the University of Florida Institutional Animal Care and Use Committee (Z951). Fieldwork was conducted under permit FNE-97011 from the Florida Game and Freshwater Fish Commission.

Ovarian Aromatase Activity

Each day, over a 3-day period, the same numbers of fish were processed from each site. Fish were anesthetized with MS-222 (150 ppm; A5040; Sigma Chemical Company, Chicago, IL, USA), and standard length and total body mass were measured to 0.1 mm and 0.001 g, respectively. Next, fish were sacrificed and then gonad and brain were immediately removed and weighed to the nearest 0.1 mg. Brains were snap-frozen

in liquid nitrogen and stored at -70°C until they were assayed, approximately 1 month later (see "Brain Aromatase Activity," below). The number of oocytes was determined for each ovary, then the ovary was placed in 488 μL of RPMI 1640 culture media (pH 7.2, sterile filtered) (Gibco 23400-021; Life Technologies, Rockville, MD, USA) in borosilicate glass tubes. Next, 13 μL of labeled androstenedione (androst-4-ene-3,17-dione, [1β - ^3H (N)], NET-926, specific activity = 24.0 Ci/mM; Dupont NEN Life Science Products, Boston, MA, USA) was added, and each sample was incubated for 6 hr at 28°C.

After incubation, samples were centrifuged at $1,500\times g$ for 15 min at 4°C, and then 400 μL of supernatant was transferred to a new borosilicate glass tube. Into each tube, 1.5 mL of chloroform (C 298-4; Fisher Scientific, Pittsburgh, PA) was added; the mixture was pulse vortexed and centrifuged as above. Next, 200 μL of supernatant was transferred to a new tube and combined with 200 μL of charcoal dextran [5% Norit A charcoal (C170, Fisher), plus 0.5% dextran (D4751, Sigma)], vortexed for 5 sec, then centrifuged as above. From these tubes, 300 μL of supernatant was transferred to a 7-mL plastic scintillation vial to which 5 mL of scintillation cocktail (Scintiverse BD; SX 18-4; Fisher) was added and the mixture vortexed for 5 sec. Tubes were read on a liquid scintillation counter (model LS5801; Beckman Instruments, Schaumburg, IL, USA).

Brain Aromatase Activity

Brains were thawed and homogenized on ice in microfuge tubes for 5 sec using a battery-powered tissue homogenizer. Next, 485 μL of RPMI 1640 culture media (see above) was added and the brain/culture media mixture was further homogenized for an additional 5 sec. To determine total protein content, an 80 μL sample was transferred to another tube (see below for total protein protocol). Then, 11 μL of labeled androstenedione (see above) was added, and each tube was incubated as described above.

Brain aromatase activity was standardized by dividing aromatase activity by total protein (milligram per milliliter of bovine serum albumin). Total protein was determined for each brain after a standard Bradford protocol (52).

Assay Validation

Aromatase activity is proportional to the amount of tritium in the scintillation vials. It was calculated as a percentage of the total substrate added. After subtracting the non-specific tritium release, the disintegrations per minute (dpm) of the sample tubes were converted to a percentage of the total dpm

added. This percentage was multiplied by the mass of the substrate added. Extraction efficiency was determined by running tritiated water (NET-100B; Dupont NEN,) through the same protocol as described and calculating the efficiency as a percentage of the total counts tubes run in duplicate. After adjusting for the loss occurring during extraction, the values obtained represent the amount of substrate converted to tritiated water, which is proportional to the aromatase activity.

Assay was validated as follows: Sensitivity was defined as twice the mean counts per minute of the blank tubes (0.38 pmol). Specificity of the assay was determined by incubating ovaries from three reference-site females with an aromatase inhibitor [4-androstene-4-ol-3,17-dione (A5791; Sigma) dissolved in methanol (27047-4; Aldrich, Milwaukee, WI, USA) was added to the RPMI 1640 culture media to a final concentration of 100 μ M]. Mean aromatase activity was decreased 93% in the aromatase inhibitor-exposed ovaries (0.12 pmol/follicle) compared with ovaries without inhibitor (1.8 pmol/follicle). Sensitivity (1.9 pmol) and specificity (mean aromatase activity decreased 70% in the aromatase inhibitor-exposed brains) for the brain aromatase assay were determined using the same methodology as described for ovarian aromatase assay.

Statistics

Anal fin length was compared between fish collected from the Fenholloway and Econfina Rivers using an analysis of covariance (ANCOVA) with standard length as the covariate and segment number was analyzed by Mann-Whitney *U*-test. Ovarian aromatase activity (pmol/follicle) was compared using the Mann-Whitney *U*-test, and the number of follicles per ovary was compared with an unpaired *t*-test. An ANCOVA for brain aromatase activity as the dependent variable and total protein as the covariate was used to compare fish from the two rivers. All analyses were carried out using the statistical software package StatView 5.0 (Abacus, Berkeley, CA, USA). *F*-tests for homoscedasticity were performed, and where necessary, data were transformed and rechecked (53). Where variance was still heterogeneous, after transformation, nonparametric statistics were used. All data are reported as the mean \pm 1 SE, and significance was determined at $p < 0.05$. Last, all reported values are nontransformed data.

Results

Fish Morphometrics

Fenholloway River females were significantly smaller in size as both standard length

($U = 116.5$, $p < 0.0001$) and mass ($U = 178$, $p < 0.0001$) were decreased compared with Econfina fish. Females from the Fenholloway averaged (\pm 1 SE) 2.44 (\pm 0.05) cm in length and 298.4 (\pm 19.6) mg in mass, whereas females from the reference river, the Econfina, averaged 3.3 (\pm 0.08) cm in length and 728.97 (\pm 88.46) mg in mass. There was no mean difference in the number of follicles with Fenholloway females [33.7 (\pm 2.8)] and Econfina females [35.8 (\pm 3.1)] ($t = -0.493$, $df = 1, 64$, $p = 0.62$). Anal fin length was not different between the two sites ($F = 0.003$, $df = 3, 79$, $p = 0.95$), whereas Fenholloway females had more segments than females from the Econfina River ($U = 302$, $p < 0.0001$).

Aromatase Activity

Ovarian aromatase activity was examined in females from both rivers and reported as picomoles of activity per follicle. Because of the presence of embryos in the ovaries of these viviparous fish, expressing aromatase activity per milligram of ovary weight would be inappropriate. An initial *F*-test for aromatase activity indicated that the variance between these two data sets was significantly heteroscedastic ($F = 7.98$, $p < 0.0001$). Arcsine transformation had no effect on the heteroscedastic nature of the variance difference of these data sets ($F = 9.04$, $p < 0.0001$). Thus, the nonparametric Mann-Whitney *U*-test was performed to determine whether a significant difference existed in aromatase activity. Females from the Fenholloway River exhibited elevated ovarian follicular aromatase activity when compared with females from the Econfina River ($U = 260.0$, $p = 0.0039$) (Figure 1).

Female fish from the Fenholloway River exhibited elevated brain aromatase activity when compared with fish from the reference site ($F = 14.4$, $df = 1, 64$, $p = 0.0003$) (Figure 2). No significant difference in the variance of brain aromatase activity was observed ($p = 0.38$) between the two populations.

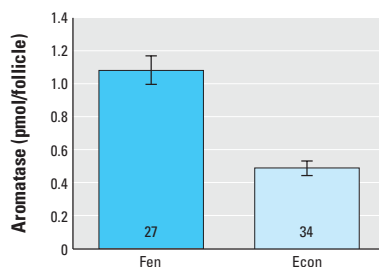


Figure 1. Ovarian aromatase activity (mean \pm 1 SE, pmol/follicle) in female mosquitofish collected from the Fenholloway (Fen, $n = 27$) and Econfina (Econ, $n = 34$) Rivers, Florida, USA. Sample size is given at the bottom of each bar. Populations exhibited a significant difference in ovarian aromatase activity ($p = 0.0039$).

Discussion

Environmental chemicals of natural and synthetic origin can interact with the endocrine system and alter development and reproduction in wildlife and humans. Most of our understanding has come from studies examining receptor-based estrogenic or antiandrogenic compounds or complex mixtures. Given the plethora of synthetic agricultural and industrial compounds that are used and eventually find their way into the environment, it is reasonable to predict that some of these could interact agonistically or antagonistically in other receptor-based signaling pathways as well as through other mechanisms such as altering rates of hormone degradation and storage. Indeed, two examples of environmental androgens have been documented: the masculinization of *a*) gastropods via tributyl tin exposure in Europe and the United States (54–56) and *b*) female mosquitofish and other fish exposed to paper mill effluent in Florida, USA (44,45,57).

In this study we tested the hypothesis that masculinization of female mosquitofish exposed to paper mill effluent in the Fenholloway River was due to aromatase enzyme inhibition. Microbial degradation of phytosterols (e.g., β -sitosterol and stigmasterol) commonly found in paper mill effluent has induced masculinized anal fin morphology in laboratory exposures (49,58). Other components of paper mill effluent include lignans and isoflavonoids (59). In humans, many biological effects are known for these compounds, including anticarcinogenic, bacteriocidal, and fungistatic activities for lignans (60). Although weak, inhibition of placental and preadipocyte aromatase activity in humans is associated with lignans, isoflavonoids, and flavonoids (61,62). We hypothesized an inhibition of aromatase activity resulting in decreased estrogen synthesis and a masculinized internal hormonal milieu for the exposed females. This hypothesis was not supported because, interestingly, we found elevated ovarian and brain

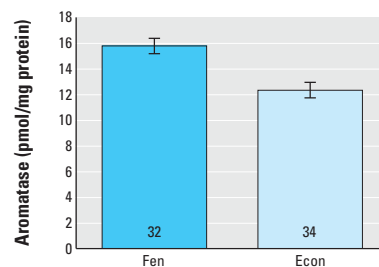


Figure 2. Brain aromatase activity (mean \pm 1 SE, pmol/mg protein) in female mosquitofish collected from the Fenholloway (Fen, $n = 32$) and Econfina (Econ, $n = 34$) Rivers, Florida, USA. Sample size is given at the bottom of each bar. Populations exhibited a significant difference in brain aromatase activity ($p = 0.0003$).

aromatase activity in the Fenholloway River females compared with that in the reference-site females.

Results of this study do not support aromatase inhibition as a mechanism of Fenholloway mosquitofish masculinization. They do suggest, however, that the masculinization is due to androgenic contaminants in the river. Although the data on androgen exposure and aromatase activity in fish are somewhat inconclusive [in one study, medaka (*Oryzias latipes*) treated with methyl testosterone exhibited decreased aromatase activity (63)], the exposure to environmental androgens is not inconsistent with increased aromatase activity. In some teleosts such as the goldfish (*Carrasius auratus*), there is an association between plasma levels of aromatizable androgen, brain aromatase activity, and aromatase mRNA at the beginning of the reproductive season (64). Further, goldfish treated with high doses of aromatizable androgen had increased plasma E_2 , supporting the relationship between increased plasma androgens and aromatase activity (65).

Through collaborative studies, we know that there are androgenic substance(s) in the Fenholloway River (15). The Fenholloway River downstream of the Buckeye Paper Mill is a highly impacted water body. Female mosquitofish living there have masculinized anal fin development and are smaller in length and mass. Although controversial, some research has suggested that exposure to the paper mill effluent masculinized the behavior of the exposed females (44), whereas other studies have shown a lower level of aggressive reproductive behavior in experimentally exposed fish (46). Although smaller in size, masculinized with respect to anal fin morphology and possibly behavior, and having altered ovarian and brain aromatase activity, female mosquitofish from the Fenholloway River still produce embryos. However, we do not know if these fry are viable or if there are transgenerational effects from exposure to paper mill effluent in these fish. We suggest that female mosquitofish are struggling to maintain an internal hormonal milieu while bathed with external environmental androgenic substances downstream of the paper mill effluent. These female fish exhibit resilience, which allows them to persist when other species have gone locally extinct.

Although individuals are known to have differing abilities in reproduction or responsiveness to environmental perturbation, few studies have examined the role of variation in maintaining resilience (66). Resilience theory has great potential in helping us understand the influences of sublethal environmental contamination (67). Resilience theory has

provided a powerful tool for ecologists to "explain" the stability and persistence of relatively complex ecosystems. Further, the models have allowed the examination of the relative stability of "alternate states" and thereby provide a means for determining levels of resilience in populations (68). It is a given that change will occur in an organism's physiology as their environment changes, but how those changes relate to population stability over time is still a question. The mosquitofish of the Fenholloway River are clearly influenced by their environment and exist in an altered state. Although the females exhibit masculinization, they survive and apparently maintain the ability to reproduce. Further studies are required to determine whether the elevated aromatase reported in this study is enough to allow these females to overcome an adverse environment and help maintain a viable population. This study, as with the many others performed during the last decade, has clearly demonstrated the complexity of trying to predict ecosystem or population health when wildlife populations experience sublethal but detrimental impacts of chemical exposure via endocrine disruption.

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